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SHORT NOTE

Derivation of Germline Competent Embryonic Stem Cells with a Combination of Interleukin-6 and Soluble Interleukin-6 Receptor

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In this report we document the derivation of pluripotential embryonic stem (ES) cells in the absence of a feeder layer by supplementation of culture media with either ciliary neurotrophic factor or oncostatin M, or with a combination of interleukin-6 (IL-6) plus soluble interleukin-6 receptor (sIL-6R). These factors all activate gp130-associated signaling processes, as does the previously characterized ES cell maintenance factor Differentiation Inhibiting Activity (Leukemia Inhibitory Factor). In particular, the IL-6/sIL-6R complex is thought to act exclusively through gp130. All ES cell lines derived using IL-6/sIL-6R contributed extensively to chimeras and were transmitted through the germline at high frequency. These findings point to a pivotal role for gp130 in ES cell propagation and may be relevant to attempts to derive ES cells from species other than mouse.

INTRODUCTION

Pluripotential murine embryonic stem (ES) cells can be propagated indefinitely *in vitro* in simple culture medium containing fetal calf serum, provided this is supplemented with the cytokine Differentiation Inhibiting Activity/Leukemia Inhibitory Factor (DIA/LIF) [1, 2, reviewed in 3]. It has also been shown that ES cells can be derived *de novo* from mouse embryos by culture in the presence of DIA/LIF [4]. The high-affinity receptor for DIA/LIF is composed of two membrane-spanning subunits, a DIA/LIF-specific component (DIA/LIF-R) and the glycoprotein gp130 [5, 6]. The latter was originally identified as a subunit of the receptor for interleukin-6 (IL-6) and is now known to be a common component of several cytokine receptor complexes [7, 8]. ES cells do not express the IL-6-specific receptor component and are therefore unresponsive to IL-6 alone. However, a soluble form of IL-6 receptor (sIL-6R) provided in combination with IL-6 has been demonstrated to interact directly with gp130 and activate signaling processes in various cell types [9]. It has recently been shown that this complex of IL-6/sIL-6R is capable of maintaining established ES cell lines in an undifferentiated state *in vitro*, apparently without involvement of DIA/LIF-R [10]. This indicates that the signaling pathway which directs self-renewal of pluripotential embryonic stem cells can be initiated through gp130 alone, with no essential requirement for DIA/LIF-R-specific signal transduction. In the present study, we confirm and extend these findings by demonstrating, first, that ES cell lines can be established *de novo* in the presence of IL-6/sIL-6R and, second, that these cell lines fulfill the definitive criterion for embryonic stem cells, which is the capacity for germline transmission. We further show that two other cytokines, oncostatin M (OSM) and ciliary neurotrophic factor (CNTF), which activate the DIA/LIF-R/gp130 receptor complex [7, 8] are also competent to support ES cell derivation. The potential relevance of these findings to the isolation of ES cells from other species is discussed.

MATERIALS AND METHODS

Mice of the 129/Ola strain were used as embryo donors. Implantation-delayed blastocysts were isolated 4 days after ovariectomy, which was performed on the third day of pregnancy. Embryos were put into culture essentially as described [4], except that the basal medium was Glasgow modification of Eagle's containing 15% fetal calf serum. Medium was supplemented with 10 ng/ml DIA/LIF (murine), 500 ng/ml of IL-6 plus 5000 ng/ml of sIL-6R (human), 50 ng/ml OSM (human), or 10 ng/ml CNTF (rat). Recombinant CNTF and OSM were obtained from Genzyme and purified recombinant DIA/LIF, IL-6, and sIL-6R were as described [10]. After 5 days, outgrowths were individually disaggregated and replated in single wells in media with the appropriate factor(s). Resultant ES cell colonies appearing in the cultures over the next 14 days were expanded through two passages into 25-cm^2 flasks in the continuous presence of the relevant factor(s). Subsequently all cultures were maintained with DIA/LIF. Cells between passages 4 and 6 were used for microinjection into C57B1/6 blastocysts. Resultant chimeras were tested for

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their ability to transmit ES cell-derived germ cells by breeding with MF1 albino mice.

RESULTS

Blastocysts plated in nonsupplemented medium in the absence of a feeder layer differentiate and do not give rise to ES cells [4, and unpublished data]. In contrast, cell lines can readily be established in the presence of DIA/LIF. The data summarized in Table 1 show that cell lines can also be derived in the presence of OSM or CNTF. Furthermore, cell lines can be isolated using IL-6/sIL-6R and with a frequency comparable to their derivation in the presence of DIA/LIF. Each of the cell lines isolated exhibited the characteristic morphology of ES cells, growing as tightly packed colonies of small cells with indistinct cell boundaries and a high nuclear to cytoplasmic ratio. The cell lines were dependent on the continuous presence of supplementing cytokines for their propagation: on withdrawal of factor the ES cells rapidly differentiated. However, all the cell lines could be maintained as undifferentiated cultures in the presence of DIA/LIF, regardless of the method of original derivation.

As summarized in Table 2, all the cell lines produced chimeras following injection into host blastocysts. In general, chimerism, as judged by coat color contribution, was extensive and compared favorably to that obtained following injection of a clone derived simultaneously in DIA/LIF. To date, test breeding of chimeras produced with cells derived in IL-6/sIL-6R has shown that all six cell lines are competent for germline transmission. Four of the five lines obtained with OSM also gave germline chimeras. These results establish unequivocally that the derived cell lines are pluripotential ES cells.

DISCUSSION

It has recently been reported that in addition to DIA/LIF, the cytokines CNTF and OSM or the combination of IL-6 plus sIL-6R are capable of maintaining established ES cells in culture [10, 11]. The finding that these factors are also competent to support the *de novo* derivation of ES cells indicates that factor responsiveness is not a consequence of long-term propagation *in vitro*, but is a property of primary embryo cultures. The extent to which this is reflective of cytokine dependency *in vivo* remains to be determined. There is clearly considerable potential for compensatory activity by different factors which may account for the viability of mutants generated by targeted mutagenesis of individual cytokine genes.

All of the factors currently known to support ES cell self-renewal act through transmembrane receptor complexes which include gp130. The demonstration that ES cells isolated using IL-6/sIL-6R are capable of germline transmission provides further evidence of a central role for the gp130 signaling system in maintenance and propagation of the pluripotential phenotype.

ES cells can readily be established from mouse embryos of strains 129 or C57BL/6, but their isolation from other strains of mice or from other species remains problematic [3]. The reason(s) for this is unclear. One possibility, however, is that embryo cells from different strains or species are not responsive to the same cytokines. In this regard it is conceivable that the activity of the common signal transducer gp130 may be more widely conserved than that of ligand-specific receptor components such as DIA/LIF-R or the CNTF receptor-α subunit. The consequent possibility that direct activation of gp130 may provide a generic route for the isolation of ES cells is under investigation.

TABLE 1

ES Cell Derivation

Factor	Number of blastocysts	Number of cell lines
DIA/LIF	14	5
OSM	12	5
CNTF	11	1
IL-6/sIL-6R	20	6

Note. Each cell line is derived from a separate blastocyst outgrowth.

TABLE 2

Chimera Production and Germline Transmission

Cell line	Blastocysts transferred	Liveborn offspring	Chimeras		Germline chimeras
			Total	Male	
IL-6A	27	9	8	8	6
IL-6B	20	11	8	7	6
IL-6C	20	11	5	3	3
IL-6D	20	5	3	0	1
IL-6E	20	9	6	5	5
IL-6F	20	8	4	1	1
DIA3	20	12	4	2	1
OSM1	22	9	7	4	4
OSM2	22	16	10	6	2
OSM3	22	10	1	0	0
OSM4	22	15	5	5	4
OSM5	20	10	4	1	2
CNTF1	20	8	0	1	0*

* No litters sired.

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